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Hematological and Blood Metabolites adaptations in Catfish *Clarias batrachus* in the Conditions of Hypoxia

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Abstract

Low oxygen concentration occurs in a wide range of aquatic systems and range in temporal frequency, seasonality and persistence. These have always been naturally occurring low oxygen habitat but anthropogenic activities related primarily to organic and nutrient enrichment have led to increase in hypoxia and anoxia both in fresh as well as marine system. Freshwater systems are more frequently faced with low oxygen condition and fishes in a tropical country like India are quite frequently exposed to this. The general public is aware of the results of hypoxia as the phenomenon of "Fish Kills" occurring frequently in natural waters.

Aquatic organisms which are frequently exposed to hypoxia show adaptations at behavioural, morphological and physiological levels. To assess the effect of hypoxia at physiological level, change in hematological and blood parameters in selected tissues of cat fish, *Clarias batrachus* was undertaken. Fish were exposed to experimentally provoked hypoxia for different duration and were sacrificed to study the effect of hypoxia on selected blood parameters in heart, liver, brain and muscle. Significant changes were recorded. The observations indicate that different tissues respond differently to the stress of hypoxia and the blood parameters respond in a tissue specific manner.

Keywords: Hematology, Blood metabolites, Catfish, Hypoxia, Glucose, Lactate.

Introduction

Hypoxia is a frequently occurring environmental phenomenon in the freshwater and even coastal system of a tropical country like India. It may be naturally occurring phenomenon due to biological and physical factors (Rosenberg *et al.*, 1991; Pihl *et al*., 1992; Hobak and Barnhart, 1996; Wu, 1999) or may be caused due to anthropogenic activities around the water bodies.

The link between hypoxia and fish responses combines behavioural and physiological strategies that can mitigate the effect of exposure to hypoxia. It may limit the energy budget or scope of growth and activity of an organism. In general the responses may be manifested at three levels:

1. Behavioural to avoid hypoxic areas, utilize well aerated micro environments or reduce activity (Kraemer, 1987; Van den Thillart *et al.,* 1994; Dalla via *et al.* 1998; Wannamaker and Rice; 2000)
2. Physiological and morphological adjustments that improve the oxygen extraction and delivery to tissues (Jensen *et al.,* 1993; Sollid *et al.,* 2003)
3. Biochemical changes that increase the capacity of tissues to function and survive at low oxygen (Hochachka, 1980; Van den Thillart and Van Waarde, 1985; Hochachka and Somero, 2002).

The first responses of fishes to environmental hypoxia are always related to respiratory and circulatory changes. Respiratory adaptations are well documented for trout and carps (Jones *et al*., 1970). Many studies have been conducted by submitting the organisms, especially fishes to hypoxia in order to study intermediary metabolic processes.

By reducing their metabolic rate during hypoxia fish delay the depletion of glycogen stores as well as the accumulation of toxic levels of lactate in the body. Changes in enzyme profiles in response to hypoxia have been undertaken in different fishes, air breathing and water breathing

both (Shouberidge & Hochachka 1983; Claireaux and Dutil 1992; Sebert *et al*., 1993; Almeida-Val *et al*., 1995). Hematological parameters are considered as patho-physiological indicators and are closely related to the responses of fish to environmental and biological factors (Fernandes and Mores, 2003). The blood parameters studied include haemoglobin (Hb), Hematocrit value (Hct) and mean corpuscular hemoglobin concentration (MCHC) from whole blood and lactate and glucose from serum. These parameters have been investigated in many fishes exposed to hypoxia (Tripathi *et al*., 2013; Muusze *et al*., 1998).

Review of Literature

The low oxygen is a major stress in the environment was inferred by the extensive researches of Jones (1952). Kutty (1968) and Bushnell *et al.,* (1984) investigated the effect of chronic hypoxia on fish swimming performance and metabolism. The effect of hypoxia on swimming activity of fishes was supported by Dahlberg *et al.,* (1968), Kutty (1968), Bushnell *et al.,* (1984). Dutil and co-workers (2007) investigated swimming performance of fishes during different periods of hypoxia. Greaney *et al.,* (1980); Taylor and Miller, (2001); Pichavant *et al.,* (2003) studied the effects of chronic (weeks of) hypoxia on oxygen carrying capacity.

Gluconeogenesis has been studied extensively in the liver and kidney in various fish species (Saurrez and Mommsen, 1987). The process was studied for the first time in an Indian air breathing fish *Clarias batrachus* by Tripathi and Co-workers (2013). These workers made extensive investigations on metabolic, behavioural and hematalogical responses of this fish to experimentally provoked hypoxia in the laboratory (Tripathi *et al.*, 2013; Kumar A. & Gopesh A. 2015; Kumar A. 2016; Kumar A. 2017).

Dunn & Hochachka (1986) and Dalla Via *et al*. (1994) observed in their studies that a metabolic reorganization takes place as a result of hypoxia that tends to follow one of two generalized patterns: (i) either the rate of anaerobic ATP production increases (Pasteur effect) or (ii) the ATP rate declines (metabolic depression).

Release of tissue specific enzyme into circulation and changes in haematological parameters have been observed in *Heteropneustes fossilis* when exposed to toxic environment (Bulow *et al.,* 1996).

Aim of the Study

Because of the link between urbanization and increased anthropogenic activities and the increase in their adverse effect on aquatic system there is a need to understand:

1. How these stresses (Hypoxia, temperature, hypercapnea, starvation etc.) in the present references affect the fish species of different respiratory habits.
2. If prolonged exposure to sub-lethal conditions can actively import coping mechanisms that are useful for the survival of these fishes.
3. The mechanisms behind the observed effect of hypoxia and improved hypoxia tolerance.

The present piece of work aims to analyze the response of blood and haematological parameters to different degrees of hypoxia in Cypriniforms, mainly catfishes, which present different respiratory patterns.

Materials and Methods

Live specimens of *Clarias batrachus (80-90 g 14-16 cm),* were procured from a local market and were acclimatized at normoxia (7.2±0.3 mg/L, DO), at least for a month in tanks of 100 L capacity filled with 25 L of water at 25±3°C. They were fed once a day with processed feed of goat liver or flesh and soybean powder. Feeding was stopped 48 h before the start of experiment. All the fishes held for 12 hrs duration of experimentally provoked hypoxia at three different levels:

1. 65%-40%Oxygen saturation or 5.0±0.3 mg/l to 3.5±0.3 mg/l O_2 (Slight Hypoxia)
2. 40%-20% Oxygen saturation or 3.5±0.3 mg/l to 1.5±0.1 mg/l O_2 (Moderate Hypoxia) and
3. Below 20%Oxygen air saturation or ≤1.5±0.1 mg/l O_2 (Severe Hypoxia)

Three separate experiments were carried out in the closed respirometer (without access to air-breath) for collection of different tissues. Decrease in dissolved oxygen (DO) was accomplished by bubbling nitrogen directly into the water of the experimental tank, or into the reservoir that supplied water to the respirometer. DO probe (WTW, CellOx 325) and pH meter (pH electrode; WTW, SenTix® 41-3) were installed to record dissolved oxygen (DO) and temperature.

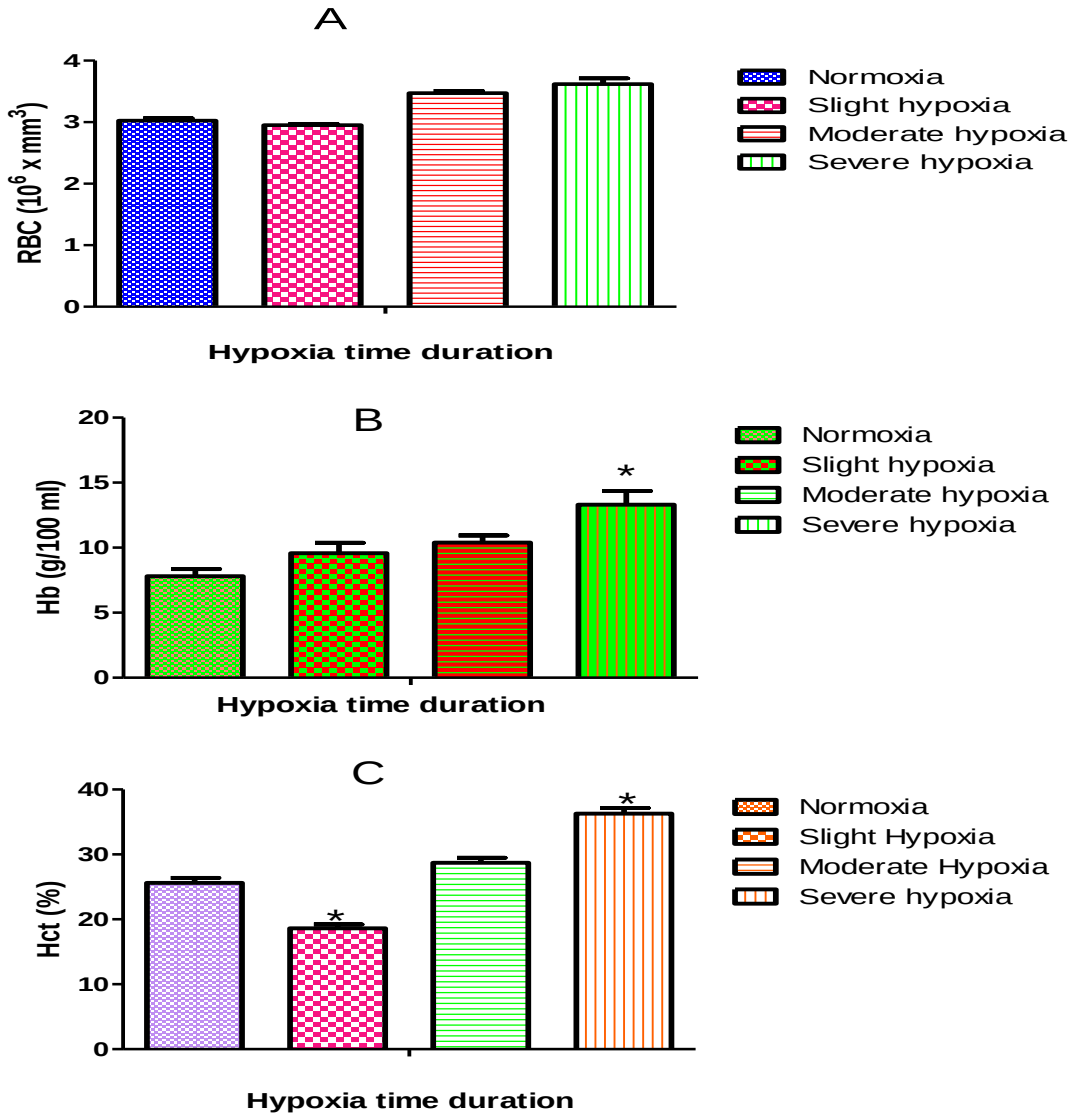
Heparinized blood was used for erythrocyte counts, haemoglobin estimation and haematocrit (Hct) evaluation. Erythrocyte count was made with the help of Neubaur's haemocytometer using standard diluents. Haemoglobin was estimated by the method of Blaxhall and Daisley (1973). [Hct] was determined following centrifugation of microhematocrit capillary tube filled with blood, at 10,000 rpm for 5 min (Assendelft and England 1982). Erythrocytic indices like mean corpuscular volume (MCV) mean corpuscular haemoglobin (MCH) Mean cell haemoglobin concentration (MCHC) was measured by Wells and Weber (1991).

Effect of Hypoxia on Blood Parameters
Clariasbatrachus

Table-1: **Haematological Changes in *Clarias Batrachus* Exposed to Different Level of Hypoxia. Values Are Mean of Three Replicates±Standard Error of Mean.**

	RBC (10^6×mm^3)	Hb(g/100ml)	Hct (%)	MCV (fl/cell)	MCH Pg/cell	MCHC (%)
Normoxia	3.026±0.039	8.8±0.55	25.6±0.77	224.49±4.13	51.25±1.65	39.09±0.42
Hypoxia						
Slight hypoxia	3.21±0.021	9.57±0.81	27.6±0.62	243.26±4.1	59.2±1.4	36.76±0.79
Moderate hypoxia	3.47±0.037	10.4±0.55	28.7±0.79	245.12±4.5	65.39±1.27	33.23±0.37
Severe hypoxia	3.62±0.091	13.31±1.05	36.27±0.89	255.45±5.6	67.52±1.74	32.11±0.32

1. There was slight decrease (2.51%) in RBC content was observed during slight hypoxia. At moderate hypoxia an increase (14%) in RBC content was observed as oxygen level in the water was decreased. It was further increased (19.62%) at severe hypoxia level (Table 1).
2. Blood haemoglobin (Hb%) was increased during all stages of hypoxia. It was increased (21.79%) at slight hypoxia and increased (33.33%) at moderate hypoxia. As water oxygen level was decreased at severely level Hb content in blood was increased (70.67%) significantly (p≤0.05%) with respect to normoxia.
3. Haematocrit (Hct%) value decreased (27.34%) at light hypoxia and increased at moderate and severe hypoxia (41.67%) significantly (p≤0.05%).
4. Other haematological parameters like MCH and MCV were also increased at all the three stages of hypoxia. MCHC increased at slight hypoxia level but decreased at moderate and severe hypoxia level (Fig. 1).



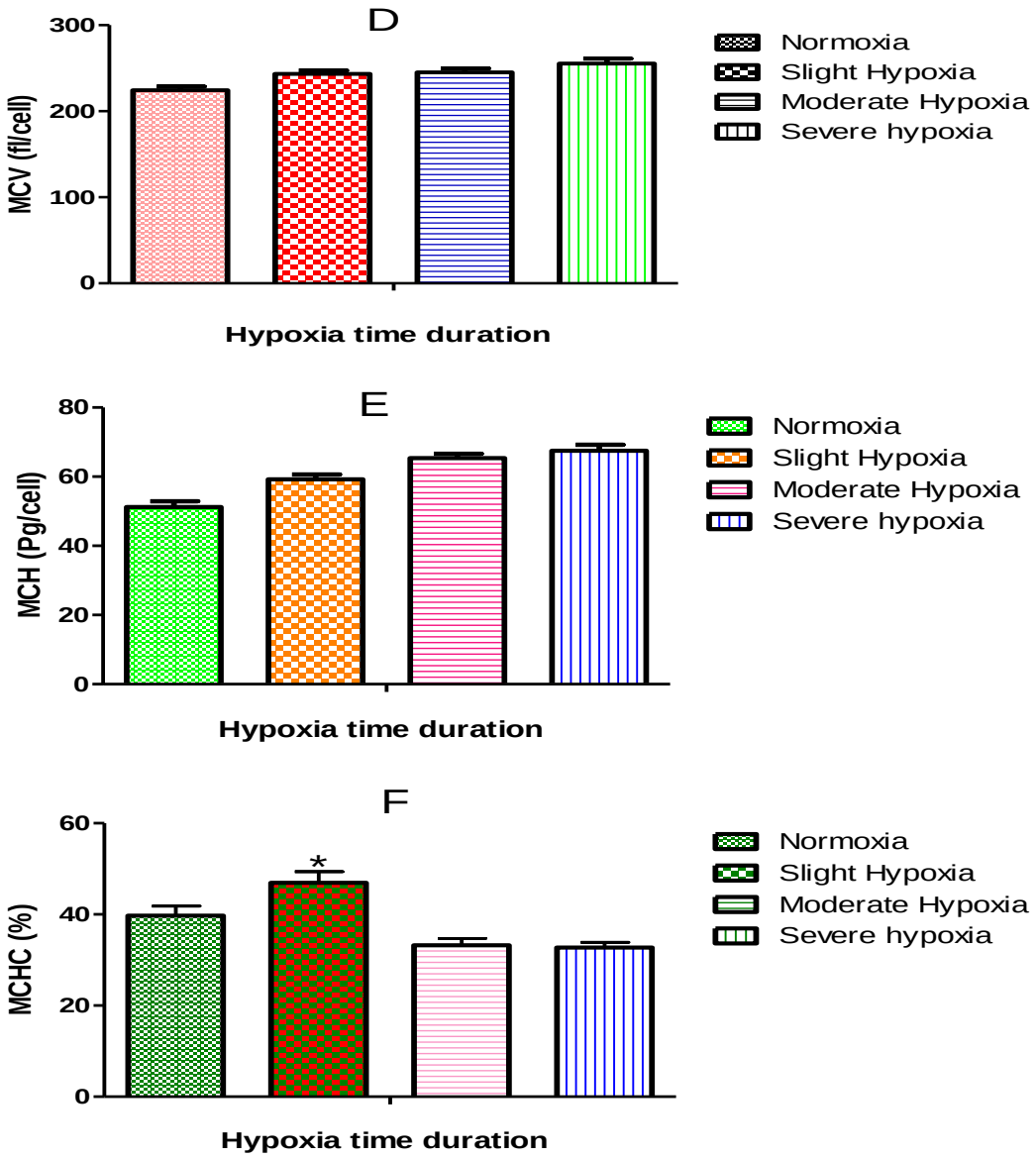


Figure-1: Haematological Parameters In Blood Of *Clarias Batrachus* Exposed To Varying Oxygen Concentration I.E. Different Hypoxia Stages For 12 Hours Duration.(A) Rbcs (10^6×Mm^3), (B) Hb (Gm/100 Ml), (C) Hct (Per Deciliter), (D) MCV (Fl/Cell), (E) MCH (Pg/Cell) And (F) MCHC (Gm/Decilitre).Asterisk (*) Represents Significant Differences (*P*<0.05) Between Normoxia And Different Hypoxia Stages.

Effect of Hypoxia on Blood Metabolite in *Clarias batrachus*

Glucose content in different tissues of Clarias batrachus

Table-2: **Determination of Tissue Glucose Content in Different Tissues of *Clarias Batrachus* Subjected to Slight, Moderate and Severe Hypoxia for Same Time Duration (12h)**

Tissues	Normoxia	Slight Hypoxia	Moderate Hypoxia	Severe hypoxia
Heart	1.42±0.15	1.23±0.14	1.12±0.12	1.63±0.14
Liver	2.43±0.16	2.25±0.21	2.15±0.19	2.68±0.29
Brain	1.65±0.13	1.72±0.33	1.56±0.21	1.31±0.16
Muscle	2.30±0.22	2.06±0.18	1.69±0.18	2.12±0.17
Blood	1.46±0.14	1.32±0.11	1.02±0.09	1.89±0.18

1. Highest glucose content was observed in liver followed by muscle and brain and lowest glucose content was observed in heart followed by blood (Table 2).
2. Normally there was a decreasing trend during slight and moderate hypoxia when compared with normoxia but it was observed to be increased

slightly at the severe hypoxia stage when compared with normoxia.

3. During slight hypoxia maximum decrease in glucose content was observed in heart (15.44%) followed by muscle (10.43%).
4. During moderate hypoxia maximum decrease in glucose content was observed in blood (30.13%) followed by heart (26.78%) and muscle (26.52%).
5. During severe hypoxia decrease in glucose content was observed in brain (20.60%) and muscle (7.82%) while an increase in glucose content probably due to gluconeogenesis and subsequent release of glucose in blood stream was observed in blood (29.45%) and heart (12.88%) followed by liver (10.28%) and.
6. Significant changes (p≤0.05%) were observed between normoxia and moderate hypoxia in heart and blood (Fig. 25).

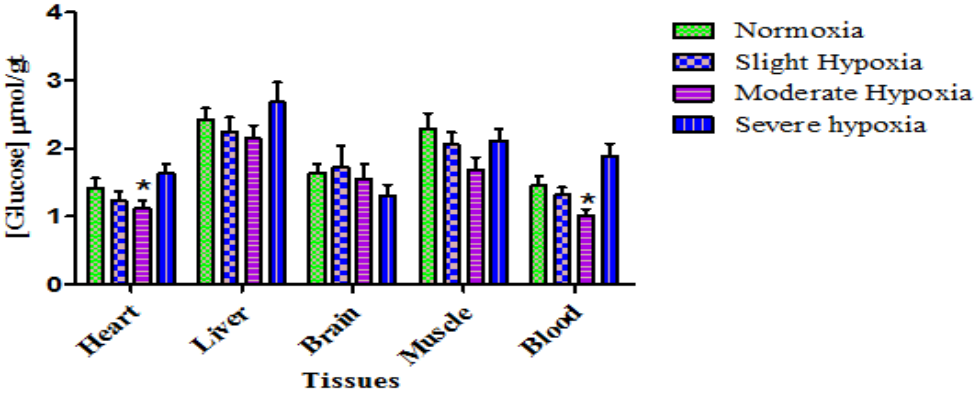


Figure-2: Glucose Concentrations In Different Tissues Of *Clarias Batrachus* Submitted To Normoxia And Different Periods of Hypoxia. Error bars are within limits of symbols when not visible. Values are means ± SD, n = 6. * p< 0.05.

Lactate content in different tissues of Clarias batrachus

Table-3: Determination of Tissue Lactate Content (µmol/Gt) in Different Tissues of *Clarias Batrachus* Subjected to Slight, Moderate And Severe Hypoxia for Same Time Duration (12h)

Tissues	Normoxia	Slight Hypoxia	Moderate Hypoxia	Severe hypoxia
Heart	0.80±0.08	1.13±0.10	1.420±0.12	1.52±0.12
Liver	0.25±0.06	0.28±0.02	0.333±0.04	0.41±0.038
Brain	0.84±0.11	1.20±0.11	1.250±0.14	1.29±0.130
Muscle	1.63±0.15	1.74±0.15	1.890±0.21	2.30±0.220
Blood	1.92±0.18	2.08±0.19	2.250±0.23	2.41±0.230

1. During normoxia highest lactate accumulation was observed in blood and muscle followed by brain and lowest lactate content was observed in liver (Table 3).
2. During slight hypoxia all tissues showed increasing trend in lactate accumulation as the fish rely mostly upon anaerobic respiration for its energy requirements and metabolic depression. Maximum increase was observed in brain (42.85%) and heart (41.25%) followed by liver (12.00%).
3. At the moderate hypoxia stage, maximum increase in lactate content was observed in heart (77.5%) followed by brain (48.8%).
4. During severe hypoxia maximum increase in lactate content was observed in Heart (90.0%), liver (64.0%) and brain (53.57%).
5. Significant changes (p≤0.05%) were observed between normoxia and moderate and severe hypoxia in heart, brain, muscle and blood (Fig. 3).

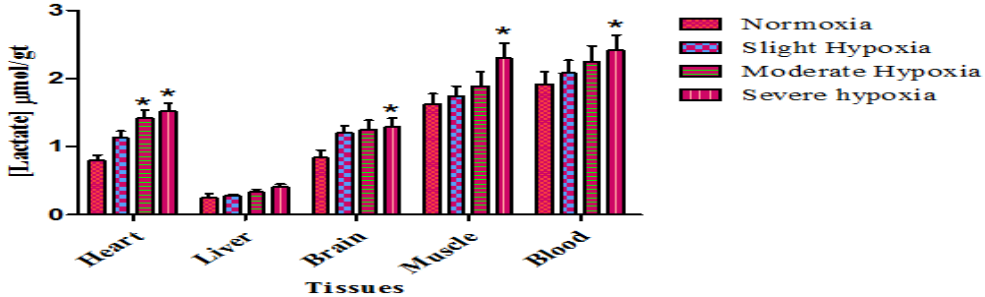


Figure-3: Lactate Concentrations In Different Tissues Of *Clarias Batrachus* Submitted To Normoxia And Different Periods of Hypoxia. Error Bars Are Within Limits Of Symbols When Not Visible. Values Are Means ± SD, N = 6. * P< 0.05.

Discussion

In the present study on air-breathing catfishes *C. batrachus*, an increase in [Hb] and [Hct] and decrease in MCHC in hypoxic conditions with mean values of [Hct] after moderate and severe exposure to hypoxia, suggested the possibility that oxygen carrying capacity of the blood might be enhanced by bringing more red blood cells into circulation. These cells are most likely released from the spleen upon adrenergic and/or cholinergic stimulation (Nilsson and Grove, 1974). These hormones serve to increase the transfer of oxygen across the gills and the transport of oxygen, in the blood, to actively metabolizing tissues. During environmental hypoxia, catecholamines are mobilized into the blood when the arterial oxygen content significantly decreases (Perry and Reid, 1974). Evidence from teleost fish suggests that the release of red blood cells via splenic contraction does occur in response to elevated catecholamines (Nilsson *et al*.1975). Splenic contraction has been well characterized in fishes in response to hypoxia (Lai and Todd, 2006).

Extended holding of large mouth bass, *Micropterus salmoides* at low DO induced an improved ability to transport oxygen in blood relative to fish held at higher oxygen concentrations. Concentrations of both Hct and Hb were significantly higher in *Micropterus salmoides* held at low oxygen for 50 days relative to fish held at higher oxygen. Hct is the percentage of packed red blood cells relative to the whole volume of blood, but does not account for the size or number of erythrocytes. Hb is a quantification of the O_2 binding protein found in red cells, whereas MCHC is a measure of the Hb in a given volume of packed erythrocytes (Houston, 1990). Increases in Hct and/or Hb are typically caused by an increase in the production of erythrocytes, swelling of the erythrocytes, or a combination of both. These changes are typically a result of catecholamine releases that induce the release of erythrocytes from the spleen (Jensen *et al*. 1993), or acidosis in the blood, which alters the affinity of Hb to bind oxygen, and can stimulate an increase in erythrocytes (Wells, 2009). Increases in Hb and Hct concentrations between the air-breathing and non air-breathing groups during an oxygen challenge may have been driven by the release of erythropoietin, the hormone responsible for synthesizing erythrocytes and releasing erythrocyte stores from the spleen. This is evidenced by the increase of erythrocytes numbers (i.e., increase in Hct and Hb) without increasing the amount of Hb per cell volume (i.e., no change in MCHC). This is only offered as a potential mechanism as erythropoietin was not quantified. Rainbow trout (*Oncorhynchus mykiss*) subjected to sustained hypoxia (maximum 216 h) had persistent increases in erythropoietin, as well as increased Hb levels (Lai *et al*.2006), thereby providing an improved ability for oxygen uptake. Additionally, long-term exposure to hypoxia increases both Hb and Hct concentrations for numerous fish species, both air and water breathers (Scott and Rogers, 1981; Tun and Houston, 1986; Petersen and Petersen, 1990 and Timmerman and Chapman, 2004). These changes typically confer an increase in oxygen-binding affinity or increased substrata for oxygen binding on the erythrocyte, improving performance of fish in low oxygen conditions. Despite Hct and Hb concentrations not differing between control treatments for these two groups, *C. batrachus* and *H. fossilis* acclimated to a low oxygen environment were able to increase those hematological variables relative to the high oxygen group following a low oxygen challenge. It is likely that this increase in Hb and Hct provided an increase in performance during hypoxia, but additional work measuring blood gas concentration and/or Hb/O2 affinity would be necessary to confirm this (Kumar A. & Gopesh A. 2015; Kumar A. 2016; Kumar A. 2017).

However, based on previous research, the prolonged exposure of *C. batrachus* (Tripathi *et. al.,* 2013) to a low oxygen environment may have conferred a beneficial advantage by improving the fish's ability to transport oxygen via erythrocytes (e.g., more red blood cells means increased surface area to bind oxygen) when exposed to an oxygen challenge.

Even though most of the enzymes involved in glucose metabolism have been detected in fish, the regulation of carbohydrate metabolism differs in some aspects from that of mammals (Moon and Foster, 1995). The regulation of hepatic glucose metabolism in teleost fishes is influenced by different stressful conditions, such as nutritional status of carbohydrates and proteins (for review, see Enes *et al*. 2009) and changes in hepato cellular hydration status (Goswami and Saha, 1998; Goswami *et al*. 2004). Gluconeogenesis has been extensively studied in the liver and kidney in various fish species (Saurez and Mommsen, 1987). In some teleostean fishes, gluconeogenesis occurs at relatively higher rates (Hayashi and Ooshiro, 1979; Renaud and Moon, 1980) and is thought to be a key process in maintaining glucose homeostasis (Carneiroand Amaral, 1982), especially in carnivorous fishes that have high protein and low carbohydrate diets (Dela Higuera and Cardenas, 1986).

In fish, increase in blood glucose level and decrease in liver glycogen level, are one of the first signs of stress and carbohydrate metabolism (Wepener, 1990). Stress response in fish is generally characterized by an increase in adrenalin causing mobilization of liver glycogen into blood glucose (Swallow and Flemming, 1970). Cortisol lowers the liver glycogen and increase in blood glucose during stress. Metabolic consequence of cortisol impairment may be a reduced capacity to mobilize liver glycogen stores (Hontela *et al*. 1995).

Carbohydrate metabolism mainly concerns to fulfill demands of animals by its aerobic and anaerobic segments (Nelson and Cox, 2002). The lactate levels acts as an index of anaerobiosis, which was beneficial for animal in tolerating hypoxic condition.

Under stress condition, with the increase of lactate content there was a decrease in pyruvate content, which suggests a shift towards anaerobiosis as a consequence of hypoxia, leading to respiratory distress (Sambasiva Rao, 1999). Blood lactic acid is

widely used as a biomarker in anoxia and pollutant stress (Srivastava and Singh, 1981).The increase in tissue lactate content is attributed to its involvement in osmoregulation (Sambasiva Rao, 1999).

There is no significant lactate accumulation in white muscle after slight hypoxia. According to Jorgensen & Mustafa (1980) significantly higher values of lactate in muscle are only registered after 21 hours of hypoxia in flounder *Platichtys flesus*. The other tissues and blood show a significant increase in lactate after up to moderate hypoxia and then a drop after severe hypoxia. Increase in lactate after hypoxia denotes an increase in anaerobic metabolism as a source of energy. Lactate produced under hypoxia may be transferred to the blood and other tissues and even kept to be oxidized after return to normal conditions. The drop in rate of increase in lactate observed in severe hypoxia in all tissues except for muscle may be due to aquatic surface respiration (ASR) that these fishes perform, especially after moderate hypoxia (Rantin & Kalinin, 1996; Rantin *et al*., 1998). Muscle and brain do not show variations between hypoxia and normoxia. Farrel & Steffensen (1987) estimated that blood lactate oxidation can fuel approximately 20% of cardiac aerobic metabolism at rest and 100% after exercise, which is consistent with findings of Milligan & Girard (1993), showing that blood lactate is a preferred substrate for cardiac muscle metabolism.

This may indicate that, although a very small tissue, cardiac muscle has the potential to play a major role in the clearance of blood lactate.

Glucose and lactate changes during hypoxia are showed in Fig2-3 in all the *Clarias batrachus*. Blood did not show significant change in glucose concentrations during hypoxia, which explains the increases and decreases of this metabolite within the tissues only. Liver showed a sharp decrease after four hours of hypoxia and subsequent recuperation, probably due to ASR. The lack of glucose increase in liver supports the conclusion that glycogenolysis was not activated in the slight and severe hypoxia but that glucose was consumed to be re-established to normal values after this period. Muscle, heart, and brain showed significant increases in glucose after severe hypoxia probably due to glycogenolysis activation.

Dunn & Hochachka (1986, 1987), both, reported an increase in glucose after hypoxia in trout *Salmo gairedeneri*. According to Walton & Cowery (1982), carbohydrate metabolism is not believed to be a major energy source in fish, but it is reasonable to assume that its importance increases during hypoxia because of its role in activation of anaerobic glycolysis activation.

The effect of various environmental factors such as temperature, hypoxia, starvation, pollution and certain hormones on carbohydrate metabolism including gluconeogenesis in different fish species has been reported by several workers (for review, see Moon and Foster 1995). There are also reports on the influence of dietary carbohydrates on gluconeogenesis in teleosts (Enes *et al*. 2009). As such, the glucose production due to gluconeogenesis is reported to be quite high in this fish liver when compared to carps and certain other teleosts (Goswami *et al*. 2004). The glucose production by gluconeogenesis was observed to be elevated further significantly in presence of all the three substrates following the exposure of the fish to hypertonic saline environment under experimental conditions. The maximum elevation was seen with lactate, indicating that an active Cori cycle is prevailing in *Clarias batrachus*.

Conclusion

There is no significant lactate accumulation in white muscle after slight hypoxia. According to Jorgensen & Mustafa (1980) significantly higher values of lactate in muscle are only registered after 21 hours of hypoxia in flounder *Platichtys flesus*. The other tissues and blood show a significant increase in lactate after up to moderate hypoxia and then a drop after severe hypoxia (Kumar A. & Gopesh A. 2015; Kumar A. 2016; Kumar A. 2017). Increase in lactate after hypoxia denotes a increase in anaerobic metabolism as a source of energy. Lactate produced under hypoxia may be transferred to the blood and other tissues and even kept to be oxidized after return to normal conditions. The drop in rate of increase in lactate observed in severe hypoxia in all tissues except for muscle, may be due to aquatic surface respiration (ASR) that these fishes perform, especially after moderate hypoxia (Rantin & Kalinin, 1996; Rantin *et al*., 1998). Muscle and brain do not show variations between hypoxia and normoxia. Farrel & Steffensen (1987) estimated that blood lactate oxidation can fuel approximately 20% of cardiac aerobic metabolism at rest and 100% after exercise, which is consistent with findings of Milligan & Girard (1993), showing that blood lactate is a preferred substrate for cardiac muscle metabolism.

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